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Identification and genotyping of Chikungunya virus using reverse transcription polymerase chain reaction and restriction fragment length polymorphism methods

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ABSTRACT

INTRODUCTION. Chikungunya virus (CHIKV) genotyping involves sequencing fractions of genes encoding E1, E2, and nsP1 proteins or the entire genome of the virus. Available reagent kits or polymerase chain reaction protocols cannot be used for CHIKV genotyping, and nucleic acid sequencing requires expensive equipment and materials, which are not always available. Therefore, it seems promising to use a simpler and more cost-effective restriction fragment length polymorphism (RFLP) method, which has not previously been used for CHIKV genotyping.
AIM. This study aimed to investigate the possibility of using reverse transcription polymerase chain reaction (RT-PCR) and RFLP for CHIKV identification and genotyping.
MATERIALS AND METHODS. The experimental study used RNA from CHIKV strains of four genotypes, including the Asian, West African (WAf), and East/Central/South African (ECSA) genotypes, and the Indian Ocean Lineage of the ECSA genotype (ECSA-IOL). The study used RT-PCR followed by DNA restriction and restriction fragment length analysis.
RESULTS. The nsP2 gene fragment of 648 bp in length (positions 3806 to 4453) contains recognition sites for the restriction endonucleases PspEI, PvuII, and DraI. The presence or absence of these sites generates a different combination specific to each of the four CHIKV genotypes. The authors designed primers for amplification of the selected gene region and performed RTPCR and RFLP.
CONCLUSIONS. The RFLP method can be used for rapid CHIKV identification and genotyping. The method provides results within a few hours and does not require high-tech equipment.

Keywords: Chikungunya virus; reverse transcription polymerase chain reaction; restriction fragment length polymorphism; genotyping

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Идентификация и генотипирование вируса Чикунгунья с использованием полимеразной цепной реакции с обратной транскрипцией и метода полиморфизма длин рестрикционных фрагментов

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РЕЗЮМЕ

ВВЕДЕНИЕ. Генотипирование вируса Чикунгунья (ЧИКВ) проводится с помощью секвенирования участков генов белков E1, E2, nsP1 или всего генома вируса. Имеющиеся наборы реагентов или протокол проведения ПЦР не позволяют генотипировать ЧИКВ, а для секвенирования нуклеиновых кислот требуются дорогостоящее оборудование и материалы, что не всегда доступно. В связи с этим перспективным представляется применение более простого и экономичного метода полиморфизма длин рестрикционных фрагментов (ПДРФ), который не использовался ранее для определения генотипов ЧИКВ.
ЦЕЛЬ. Изучение возможности применения полимеразной цепной реакции с обратной транскрипцией и метода полиморфизма длин рестрикционных фрагментов для идентификации и генотипирования вируса Чикунгунья.
МАТЕРИАЛЫ И МЕТОДЫ. В экспериментальном исследовании использовали РНК штам-мов ЧИКВ четырех генотипов: Азиатский (Asian), Западно-Африканский (WAf), Восточно-Центральный Южно-Африканский (ECSA) и Восточно-Центральный Южно-Африканский — линия Индийского океана (ECSA-IOL). Проводили полимеразную цепную реакцию с обратной транскрипцией (ОТ-ПЦР) с последующей рестрикцией и анализом длин рестрикционных фрагментов.
РЕЗУЛЬТАТЫ. Установлено, что фрагмент гена nsP2 длиной 648 п.н. между позициями 3806 и 4453 содержит сайты узнавания эндонуклеазами рестрикции PspEI, PvuII и DraI, наличие или отсутствие которых составляют различные комбинации и специфичны для каждого из четырех генотипов ЧИВК. Сконструированы праймеры, позволяющие амплифицировать выбранный участок гена, проведены ОТ-ПЦР и анализ ПДРФ.
ВЫВОДЫ. Для быстрого подтверждения подлинности вируса Чикунгунья и его генотипирования может быть успешно использован метод полиморфизма длин рестрикционных

фрагментов, который позволяет провести анализ в течение нескольких часов и в отсутствие высокотехнологичного оборудования.

Ключевые слова: вирус Чикунгунья; полимеразная цепная реакция с обратной транскрипцией; метод полиморфизма длин рестрикционных фрагментов; генотипирование

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INTRODUCTION

Chikungunya virus (CHIKV), transmitted by *Aedes* mosquitoes found in tropical and subtropical countries, is the causative agent of chikungunya fever, which is characterised by an acute febrile reaction and rash followed by potentially chronic myalgia and arthralgia [1, 2].

CHIKV belongs to the *Alphavirus* genus of the *Togaviridae* family and has four genotypes, including the Asian, West African (WAf), and East/Central/South African (ECSA) genotypes, and the Indian Ocean Lineage of the ECSA genotype (ECSA-IOL). Despite their names, CHIKV genotypes do not have a clear geographical distribution pattern, and several genotypes can be isolated from the same territory [2–6].

Currently, CHIKV detection in mosquitoes and laboratory confirmation of clinical diagnosis are based on a testing algorithm developed by the US Centers for Disease Control and Prevention (CDC). The algorithm involves the use of primers for different regions of envelope glycoprotein 1 (E1), envelope glycoprotein 2 (E2), and non-structural protein 1 (nsP1) genes or, alternatively, commercial kits for real-time polymerase chain reaction (PCR) [2–10].

It is important to identify the viral strain genotype to determine the geographical source of infection during epidemiological investigations. CHIKV genotyping involves sequencing fragments of genes encoding E1, E2, and (less frequently) nsP1 proteins or the entire viral genome [1–3, 6, 8, 9, 11, 12]. However, nucleic-acid sequencing requires costly equipment and materials, which are not always available, and neither the existing test kits nor the PCR protocol can genotype the virus.

Previous findings suggest that the restriction fragment length polymorphism (RFLP) method can be used to genotype paramyxoviruses (mumps and measles) and matonaviruses (rubella); potential applications extend up to rapid identification of specific viral strains [13–15]. Therefore, it seems reasonable to suggest that RFLP applications may be used to genotype CHIKV. This will be the first use of the method for this purpose.

This study aimed to investigate the possibility of using reverse transcription polymerase chain reaction (RT-PCR) and RFLP for CHIKV identification and genotyping.

MATERIALS AND METHODS

The study used RNA of alphaviruses and flaviviruses. The *Alphavirus* genus was represented by CHIKV virus (four genotypes, Asian, ECSA, ECSA-IOL, and WAf, three strains of each, pre-identified by sequencing), Venezuelan equine encephalomyelitis virus (two strains), and Sindbis virus (two strains). The *Flaviviridae* family was represented by yellow fever virus strain 17D, dengue virus serotypes 1–4, Japanese encephalitis, West Nile virus, and tick-borne encephalitis virus (one strain of each).

The strains that were used for RNA production were obtained from the collections of the Saint Petersburg Scientific Research Institute of Vaccines and Serums and the Enterprise for the Production of Bacterial Preparations of the Federal Medical and Biological Agency of Russia (Nicaragua Branch), the I. Mechnikov Research Institute of Vaccines and Sera (Russia), and the Republican Research and Practical Center for Epidemiology and Microbiology of the Ministry of Health of the Republic of Belarus.

The study was conducted in full compliance with the current health and safety regulations.

RNA extraction. RNA was extracted from the virus samples using an AmpliSens® MAGNO-Sorb test kit (Central Research Institute for Epidemiology, Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing) according to the instructions provided by the manufacturer. RNA was extracted into 50 µL of elution solution, and the resulting samples were stored at –70 °C until further use.

Synthesis of cDNA from RNA samples. RT-PCR was performed in a final volume of 60 µL. The reaction mixture included 6 µL of SE 10x M-MuLV buffer, 0.6 µL of dNTP mix (40 mM), 1.4 µL of M-MuLV reverse transcriptase (50 U/µL), 8 µL of specific reverse primer (24 pmol/µL), 16 µL of an RNA sample, and 28 µL of water (all the reagents were produced by SibEnzyme Ltd, Russia). The reaction proceeded for 30 min at 42 ºC.

PCR amplification. DNA fragments of 648 bp were amplified in 125 µL of reaction mixture containing 25 µL of cDNA, 5 µL of each specific primer (3 pmol/µL), 12.5 µL of SE Taq DNA polymerase buffer, 25 µL of Taq DNA polymerase (5000 U/mL), 1.5 µL of dNTP mix (40 mM), and 51 µL of water (all the reagents were produced by SibEnzyme Ltd, Russia). Thermal cycling was conducted using a DTprime 5M1 amplifier (DNA-Technology LLC, Russia) and the following programme: a 90 s hot start at 95 °C, 35 amplification cycles (20 s at 95 °C, 40 s at 55 °C, 40 s at 72 °C, and 40 s at 72 °C), 10 min at 72 °C, and storage at 10 °C. The restriction analysis involved digestion with the restriction endonucleases DraI, PvuII, and PspEI (SibEnzyme Ltd, Russia). After PCR amplification, 25 µL reaction mixture aliquots were supplemented with 5 µL of ROSE restriction buffer (SibEnzyme Ltd, Russia) and 20 µL of water and then divided into two aliquots of 25 µL each. One was mixed with 1 µL of restriction endonuclease, and the other was used as a negative control. After 1 h of incubation at 37 °C, the digestion products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualised under transmitted UV light using a transilluminator. Recombinant plasmid DNA carrying a sequence encoding CHIKV nsP2 (Cat. No. D-1652, Bioservice Biotechnology Company Ltd, Russia) was used as a positive control.

Software. Multiple nucleotide alignment of CHIKV sequences was performed using MAFFT version 7[1]. Oligonucleotide primers were designed using OLIGO 4.0[2].

RESULTS AND DISCUSSION

Selection of a CHIKV genome fragment suitable for RFLP genotyping

The selection of a CHIKV fragment for RFLP analysis included the following steps:

- multiple alignment of 731 CHIKV genome sequences from the GenBank database of the National Centre for Biotechnology Information (NCBI)[3] that had their genotypes specified;
- analysis of the most conserved RNA regions of each of the four CHIKV genotypes;
- selection of the optimal fragment for CHIKV genotyping by the presence/absence of restriction endonuclease recognition sites;
- selection of specific forward and reverse primer sequences for the amplification of the selected fragment of the CHIKV genome sequences.

Sequence analysis of the CHIKV strains available in GenBank to select a CHIKV genome fragment for genotype differentiation by RFLP

A GenBank search for CHIKV nucleotide sequences of at least 10,000 bp selected a total of 731 nucleotide sequences of all four CHIKV genotypes. The search identified genotype-specific combinations depending on the presence or absence of restriction endonuclease recognition sites in a 648 bp nsP2 gene fragment located between positions 3806 and 4453 (relative to the reference CHIKV genome, GenBank NC_004162). The structural variability of RNA in this fragment informed the selection of degenerate primers suitable for RT-PCR amplification of the target genomic fragment of the studied CHIKV strains *(Table 1)*.

Multiple sequence alignment showed that the selected nsP2 gene fragment may contain recognition sites for the restriction endonucleases PspEI, PvuII, and DraI, depending on the CHIKV genotype *(Fig. 1, Table 2)*.

CHIKV genotypes differ in the PspEI, PvuII, and DraI restriction sites *(Table 2)*. Asian, ECSA, and ESCA-IOL genotype strains contain a GGTTACC sequence that is cleaved with PspEI at position 350 of the selected nsP2 gene fragment (GGTNACC recognition site), whereas WAf genotype strains do not contain this sequence. ESCA-IOL genotype strains carry a single nucleotide substitution of thymine (T) for cytosine (C) at position 252, which results in the formation of a second PspEI recognition site. Thus, the PspEI digestion of a PCR product of Asian/ECSA genotype strains should yield two fragments of 350 and 298 bp, and that of a PCR product of ESCA-IOL genotype strains should produce three fragments of 298, 252, and 98 bp. In contrast, PspEI treatment of a PCR product of WAf genotype strains should not result in cleavage. According to the analysis, the WAf, ECSA, and ESCA-IOL genotype strains carry adenine (A) instead of guanine (G) at position 527, which results in a CAGCTG sequence and a PvuII recognition site. WAf genotype strains carry another substitution—G instead of A at posi-

[1] https://mafft.cbrc.jp/alignment/server/
[2] https://www.oligo.net/
[3] https://www.ncbi.nlm.nih.gov

Table 1. Oligonucleotide structures for Chikungunya virus detection using reverse transcription polymerase chain reaction (RT-PCR)
Таблица 1. Структура олигонуклеотидов для выявления РНК вируса Чикунгунья с помощью ПЦР с обратной транскрипцией

Definition *Наименование*	**Designation** *Обозначение*	**Sequence (5'–3')** *Последовательность (5'–3')*	**Genomic coordinates (relative to the reference genome NC_004162, GenBank)** *Координаты в геноме (относительно референсного генома NC_004162, GenBank)*	**PCR product size (bp)** *Размер ПЦР-продукта, (п.н.)*
Forward primer *Прямой праймер*	CHIC2d	GG(G/A)GG(T/A)GACTC(A/C)(T/C) TGAGACTGCTCA (25 *b*/н.)	3806-3831	648
Reverse primer *Обратный праймер*	CHIC2r-2	GTT(C/T)A(G/A)TGA(C/T)TG(G/A) GT(C/T)AGCCT(G/A)TCTTT (27 *b*/н.)	4426-4453	

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tion 226—creating an additional PvuII recognition site. Asian genotype strains do not contain any PvuII recognition sites. To summarise, PvuII cannot cleave the 648 bp nsP2 gene fragment of Asian genotype strains; however, the enzyme should yield 527 and 121 bp fragments for ECSA/ESCA-IOL genotype strains and 301, 226, and 121 bp fragments for WAf genotype strains.

Some analysed CHIKV strains of the ECSA genotype (including the reference NC_004162 sequence, GenBank) lack the PvuII recognition site at position 527 of the genome sequence. Such strains can be distinguished from Asian genotype strains by amplicon cleavage with DraI. According to the results of multiple sequence alignment, the Asian genotype has a DraI recognition site at position 403 of the nsP2 gene fragment. This recognition site should provide for PCR product cleavage into three fragments of 403 and 245 bp. For the other three CHIKV genotypes, the DNA fragment contains G instead of A at position 408, and therefore the PCR product cannot be cleaved with DraI. As follows from the data summarised in *Table 2*, the restriction endonucleases PvuII and PspEI are applicable for CHIKV genotyping by the RFLP method, while the DraI enzyme can be used to distinguish between the ECSA and Asian genotypes.

Primer specificity assessment

The specificity of the selected primers was determined by RT-PCR. RT-PCR amplification with a control recombinant plasmid carrying a CHIKV nsP2 sequence produced a fragment of 648 bp in length, which corresponded to the calculated length. RT-PCR amplification with heterologous viral RNA as the matrix (see *Materials and Methods*) did not yield any amplification product. In contrast, RT-PCR amplification with RNA samples of all four CHIKV genotypes produced a fragment of the expected length, showing that the selected primers were specific to all the existing CHIKV genotypes.

Results of RT-PCR followed by PspEI, PvuII, and DraI enzymatic restriction

Practical testing of the developed RT-PCR- and RFLP-based CHIKV genotyping method involved the use of RNA from CHIKV strains of different genotypes. The PspEI and PvuII cleavage of the 648 bp fragment of the nsP2 gene yielded fragments of the expected lengths calculated for the CHIKV genotypes *(Fig. 2)*.

The restriction results were clearly visualised by electrophoresis *(Fig. 2)*. PspEI did not cleave the amplified fragment of the WAf genotype, whereas PvuII cleaved it into three fragments, showing the presence of two PvuII restriction sites. This feature differentiates the WAf genotype from the other genotypes. The study showed that there are no PvuII restriction sites in the fragment of interest of the Asian genotype. This difference enabled differentiation between the Asian and ECSA genotypes in the majority of the studied cases. Analysis of PCR products of the ECSA and ECSA-IOL genotypes demonstrated that PvuII cleaved the fragment of interest into identical fragments. In contrast, cleavage with PspEI produced distinctly different fragments.

To differentiate between the ECSA and Asian genotypes, the 648 bp fragment of the nsP2 gene was cleaved using DraI.

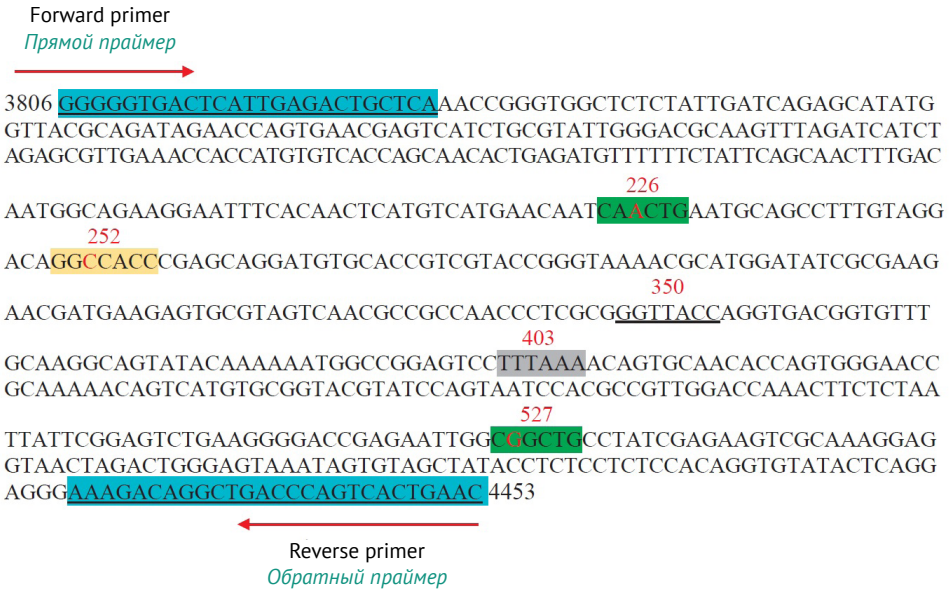

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Fig. 1. Restriction site location in the selected nsP2 gene fragment of Chikungunya virus (genome segment assembled for all the study strains).

Рис. 1. Расположение сайтов рестрикции в выбранном фрагменте гена nsP2 вируса Чикунгунья (сконструированный участок генома всех штаммов).

Note. Recognition sites for the restriction enzymes: PspEI, GGtNACC; DraI, TTTAAa; PvuII, CAgCTG. Nucleotide substitutions at the restriction sites are marked in red.
Примечание. Сайты узнавания для рестриктаз: PspEI – GGtNACC; DraI – TTTAAa; PvuII – CAgCTG. Красным цветом отмечена замена нуклеотида в сайте рестрикции.

Table 2. Estimated restriction fragment lengths in Chikungunya virus (CHIKV) typing by the restriction fragment length polymorphism method
Таблица 2. Расчетная длина фрагментов рестрикции при типировании вируса Чикунгунья (ЧИКВ) методом полиморфизма длин рестрикционных фрагментов

CHIKV genotype *Генотип ЧИКВ*	**Number / estimated length of restriction fragments (bp)** *Количество / ожидаемая длина фрагментов рестрикции (п.н.)*		
	PspEI	**PvuII**	**DraI**
WAf	One/648 *Один/648*	Three/301; 226; 121 *Три/301; 226; 121*	One/648 *Один/648*
Asian	Two/350; 298 *Два/350; 298*	One/648 *Один/648*	Two/403; 245 *Два/403; 245*
ECSA	Two/350; 298 *Два/350; 298*	Two/527; 121* *Два/527; 121**	One/648 *Один/648*
ESCA–IOL	Three/298; 252; 98 *Три/298; 252; 98*	Two/527; 121 *Два/527; 121*	One/648 *Один/648*

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Note. CHIKV genotypes: Asian, West African (WAf), East/Central/South African (ECSA), and ECSA Indian Ocean Lineage (ECSA–IOL).
* There may be no PvuII site at position 527 in the genomes of some ECSA strains (including the reference genome NC_004162, GenBank).
Примечание. Генотипы ЧИКВ: Азиатский (Asian), Западно-Африканский (West African, WAf), Восточно-Центральный Южно-Африканский (East-Central-South African, ECSA) и Восточно-Центральный Южно-Африканский – линия Индийского океана (East-Central-South African – Indian Ocean Lineage, ECSA–IOL).
* – сайт PvuII в позиции 527 в геномах некоторых штаммов генотипа ECSA (включая референсный NC_004162, GenBank) может отсутствовать.

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Fig. 2. Electrophoretic detection results for PCR fragments of the nsP2 gene of Chikungunya virus (CHIKV) strains of different genotypes, obtained upon hydrolysis with the restriction endonucleases PspEI and PvuII. Electropherogram lanes: 1–2, molecular weight markers; 3, amplified CHIKV nsP2 gene fragment (estimated length: 648 bp); 4–7, fragments of CHIKV genotypes restricted by PspEI (4, Asian; 5, East/Central/South African (ECSA); 6, ESCA Indian Ocean Lineage (ECSA-IOL); 7, West African (WAf)); 8–11, DNA fragments of CHIKV genotypes restricted by PvuII (8, Asian; 9, ECSA; 10, ECSA-IOL; 11, WAf).

Рис. 2. Результаты электрофоретической детекции ПЦР-фрагментов гена nsP2 штаммов вируса Чикунгунья (ЧИКВ) разных генотипов, полученных после гидролиза при использовании рестриктаз PspEI и PvuII. Дорожки на электрофореграмме: 1, 2 — маркеры молекулярного веса; 3 — амплифицированный фрагмент гена nsP2 ЧИКВ (расчетная длина 648 п.н.); 4–7 — фрагменты после рестрикции эндонуклеазой PspEI последовательностей штаммов, относящихся к генотипам: Азиатский (Asian) — 4, Восточно-Центральный Южно-Африканский (ECSA) — 5, ECSA линия Индийского океана (ECSA-IOL) — 6, Западно-Африканский (WAf) — 7; 8–11 — фрагменты после рестрикции эндонуклеазой PvuII последовательностей штаммов генотипов: Asian — 8, ECSA — 9, ECSA-IOL — 10, WAf — 11.

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Fig. 3. Electrophoretic detection results for DNA fragments of the nsP2 gene of Chikungunya virus (CHIKV) strains of Asian and East/Central/South African (ESCA) genotypes, obtained upon hydrolysis with the restriction endonucleases PspEI and DraI. Electropherogram lanes: 1–2, amplified CHIKV nsP2 gene fragments (estimated length: 648 bp) (1, Asian; 2, ECSA); 3–4, DNA fragments of CHIKV strain genotypes restricted by PspEI (3, Asian; 4, ECSA); 5–6, DNA fragments of CHIKV strain genotypes restricted by DraI (5, ECSA; 6, Asian).

Рис. 3. Результаты электрофоретической детекции ДНК-фрагментов гена nsP2 штаммов вируса Чикунгунья (ЧИКВ) генотипов Азиатский (Asian) и Восточно-Центральный Южно-Африканский (ECSA), полученных после гидролиза при использовании рестриктаз PspEI и DraI. Дорожки на электрофореграмме: 1, 2 — амплифицированный фрагмент гена nsP2 ЧИКВ (расчетная длина 648 п.н.) генотипа Asian (1), генотипа ECSA (2); 3, 4 — ДНК-фрагменты после рестрикции эндонуклеазой PspEI последовательностей штаммов генотипов: Asian (3), ECSA (4); 5, 6 — ДНК-фрагменты после рестрикции эндонуклеазой DraI последовательностей штаммов генотипов: ECSA (5), Asian (6).

Restriction analysis *(Fig. 3)* demonstrated that the lengths of the fragments generated by enzymatic cleavage were consistent with the calculated lengths. PspEI cleaved the amplicon of the Asian and ECSA genotypes into two fragments of 350 and 298 bp. DraI did not cleave the amplified fragment of the ECSA genotype. However, DraI cleaved the PCR product of the Asian genotype into two fragments of 403 and 245 bp. Thus, the RFLP method proved effective in differentiating between all the CHIKV genotypes studied, and the results of the restriction analysis coincided with the data that had been obtained by sequencing earlier.

CONCLUSION

The results obtained demonstrate that the CHIKV nsP2 gene contains a fragment with restriction sites whose combinations are specific for each virus genotype. As theoretically predicted and experimentally confirmed, RT-PCR followed by RFLP analysis can be used for CHIKV genotyping. The test does not require much time or high-tech equipment. In the future, the results obtained can be used to identify and genotype biological materials that may contain the Chikungunya fever pathogen.

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